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## RESEARCH ARTICLE

Coloration mechanisms and phylogeny of *Morpho* butterfliesM. A. Giraldo<sup>1,\*</sup>, S. Yoshioka<sup>2</sup>, C. Liu<sup>3</sup> and D. G. Stavenga<sup>4</sup>

## ABSTRACT

*Morpho* butterflies are universally admired for their iridescent blue coloration, which is due to nanostructured wing scales. We performed a comparative study on the coloration of 16 *Morpho* species, investigating the morphological, spectral and spatial scattering properties of the differently organized wing scales. In numerous previous studies, the bright blue *Morpho* coloration has been fully attributed to the multi-layered ridges of the cover scales' upper laminae, but we found that the lower laminae of the cover and ground scales play an important additional role, by acting as optical thin film reflectors. We conclude that *Morpho* coloration is a subtle combination of overlapping pigmented and/or unpigmented scales, multilayer systems, optical thin films and sometimes undulated scale surfaces. Based on the scales' architecture and their organization, five main groups can be distinguished within the genus *Morpho*, largely agreeing with the accepted phylogeny.

**KEY WORDS:** Wing scales, Spectrophotometry, Scatterometry, Multilayers, Thin films, Butterfly phylogeny

## INTRODUCTION

Butterflies of the Neotropics and particularly the genus *Morpho* have for centuries intrigued scientific researchers as well as laymen because of their striking colors and gracious flight (DeVries et al., 2010). Considerable insight into the evolution of *Morpho* has been gained by phylogenetic studies (Penz and DeVries, 2002; Penz et al., 2012; Cassildé et al., 2012; Blandin and Purser, 2013). Furthermore, detailed anatomical and optical investigations of the scales that cover the wings have yielded substantial knowledge of the physical basis of the brilliant-blue colored wings (Ghiradella, 1984; Vukusic et al., 1999; Yoshioka and Kinoshita, 2004; Berthier, 2007; Kinoshita, 2008).

Butterfly wing scales basically consist of two laminae connected by pillar-like structures, the trabeculae (Ghiradella, 1989). The lower lamina is commonly a smooth membrane that can act as an optical thin-film reflector (Yoshioka and Kinoshita, 2004; Stavenga et al., 2014; Giraldo and Stavenga, 2016). The upper lamina is a much more intricate structure, consisting of an array of ridges parallel to the longitudinal axis of the scale and an array of cross-ribs at right angles to the former.

The wings of butterflies are shingled on both the ventral and dorsal side by a regular lattice of overlapping ground and cover

scales (Ghiradella, 1998). The ventral wing side of *Morpho* butterflies is studded by variously pigmented scales, together creating a disruptive pattern that may provide camouflage when the butterflies are resting with closed wings. When flying, *Morpho* butterflies display the dorsal wing sides, which are generally bright-blue in color. The metallic reflecting scales have ridges consisting of a stack of slender plates, the lamellae, which in cross-section show a Christmas tree-like structure (Ghiradella, 1998). This structure is not exclusive to *Morpho* butterflies and has also been found in other butterfly species, for instance pierids, reflecting in the blue and/or UV wavelength range (Ghiradella et al., 1972; Rutowski et al., 2007; Giraldo et al., 2008). In *Morpho*, with their thickness and spacing being in the ~100 nm range, the lamellae create a multilayer that strongly reflects blue light. The number of layers, which determines the reflection intensity, varies with the species and type of scale (Gralak et al., 2001; Kinoshita et al., 2002; Plattner, 2004; Berthier et al., 2006). The multilayered scales are classified as iridescent because the reflectance spectrum depends on the angle of illumination. The margins of the *Morpho*'s blue dorsal wings are commonly brown–black, because of pigmentary colored scales containing concentrated melanin pigment (Berthier, 2007).

In our previous study, we investigated three characteristic *Morpho* species, *M. epistrophus*, *M. helenor* and *M. cypris*, and concluded that their brilliant iridescence is due to both a thin film lower lamina and a multilayered upper lamina (Giraldo and Stavenga, 2016). Here we present a comparative study on 16 of the 30 accepted *Morpho* species (Blandin and Purser, 2013; Chazot et al., 2016). We specifically focus on the spectral and morphological scale characteristics, at both a macroscopical and a microscopical level, by applying microscopy, spectrophotometry and imaging scatterometry. The assembled data indicate a distinct classification of the investigated species. We have come to identify five groups according to the overlapping of ground scales by cover scales, which appears to be in fair agreement with the recently deduced phylogeny of the Morphinae (Blandin and Purser, 2013).

## MATERIALS AND METHODS

## Animals

We studied specimens of 16 butterfly species belonging to the genus *Morpho* Fabricius 1807. *Morpho achilles*, *M. aega*, *M. cypris*, *M. deidamia*, *M. epistrophus*, *M. godarti*, *M. helenor helenor*, *M. marcus*, *M. menelaus didius*, *M. menelaus menelaus*, *M. portis*, *M. rhetenor*, *M. sulkowskyi*, *M. theseus* and *M. zephyritis* were purchased from commercial suppliers. Dr Marta Wolff, Entomology Group, University of Antioquia (Medellín, Colombia) generously supplied *M. helenor peleides*.

## Microphotography

Local areas of intact wings and single wing scales were photographed with a Zeiss Universal Microscope (Zeiss, Oberkochen, Germany) using Zeiss Epiplan objectives (8×/0.2 or 16×/0.35) and a Kappa DX-40 (Kappa Optonics GmbH, Gleichen, Germany) digital camera. Single wing scales were obtained by

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gently pressing intact wings to a glass microscope slide. The isolated scales were glued to the tip of a glass micropipette, which was mounted on the rotatable stage of the microscope.

### Imaging scatterometry

To investigate the spatial far-field reflection characteristics, we performed imaging scatterometry on small wing pieces and single scales (Stavenga et al., 2009), which were attached to the tip of a glass micropipette and positioned at the first focal point of the ellipsoidal mirror of the imaging scatterometer. Scatterograms were obtained by focusing a white light beam with a narrow aperture ( $<5^\circ$ ) onto a circular spot with diameter  $\sim 400\ \mu\text{m}$  (wing pieces) or  $\sim 30\ \mu\text{m}$  (isolated scales). The spatial distribution of the far-field scattered light was recorded with an Olympus DP70 digital camera (Olympus, Tokyo, Japan). The red circles in the scatterograms (e.g. Fig. 1I–L) indicate reflection cone angles of  $5^\circ$ ,  $30^\circ$ ,  $60^\circ$  and  $90^\circ$ . The scatterograms thus represent the far-field reflection hemisphere.

### Spectrophotometry

Reflectance spectra of intact wings were recorded with an integrating sphere (AvaSphere-50-Refl; Avantes, Apeldoorn, the Netherlands) using a deuterium-halogen lamp [Avantes AvaLight-D(H)-S] and an AvaSpec-2048 spectrometer (Avantes). A white reflectance standard (WS-2, Avantes) served as a reference. Reflectance spectra were also measured of single scales for both the abwing and adwing sides (ab=away from; ad=toward), i.e. the sides facing the observer and the wing, respectively, with a custom-built microspectrophotometer (MSP), which consists of a Leitz Ortholux epi-illumination microscope connected with a fiber-optic cable to the AvaSpec-2048 spectrometer. The light source was a xenon arc and the microscope objective was an Olympus LUCPlanFL N  $20\times/0.45$ . Owing to the glass optics, the MSP spectra were limited to wavelengths  $>350\ \text{nm}$ .

### Scanning electron microscopy

A Philips XL-30 scanning electron microscope was used to investigate single scales placed on a carbon stub holder, with either the upper or lower lamina exposed. To reveal the transversal morphology of the scales, scales were trans-sectioned with a razor blade. Prior to imaging, the samples were sputtered with gold.

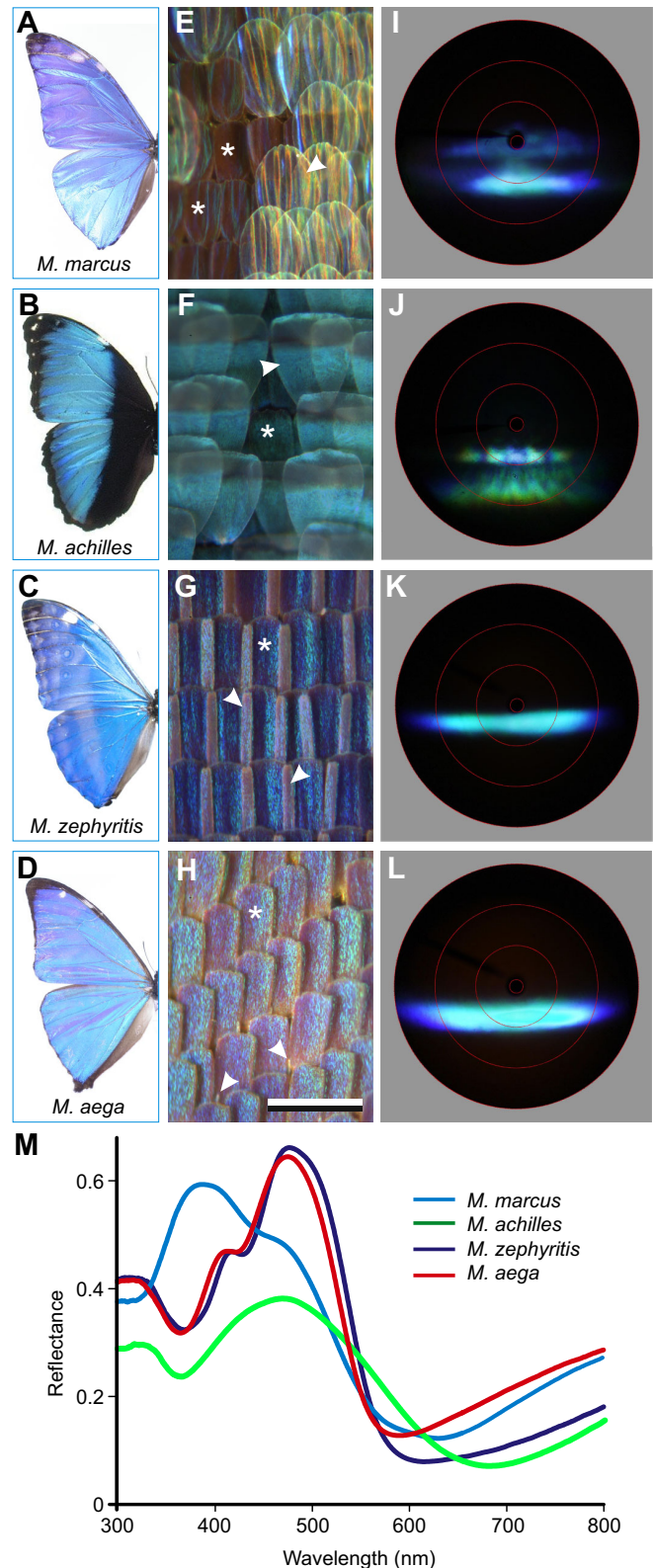
## RESULTS

### Different scale lattices of *Morpho* butterflies

We studied the dorsal wing sides of male butterflies of 16 *Morpho* species and classified for each species the lattice organization, the morphology of the scales covering the wings, their spectral and spatial optical properties, and the number of lamellae that form the ridges. The experimental results indicated a division of the investigated species into five groups.

In our previous study (Giraldo and Stavenga, 2016), we extensively described the case of *M. epistrophus*. Its unpigmented cover and ground scales, which have a blueish coloration owing to the scales' lower laminae acting as an optical thin film, appeared to be built according to the general bauplan of the related nymphaline butterflies (Ghiradella, 1984, 1989, 1998; Stavenga et al., 2014; Giraldo and Stavenga, 2016). We therefore classified *M. epistrophus* as *Morpho*'s basic group G0.

The other investigated species had distinctly different scale properties, which caused a division of these species in four distinct groups, G1–G4. We chose from each of these groups a representative species: *M. marcus*, *M. achilles*, *M. zephyritis* and *M. aega* (Fig. 1). The four selected species all have brilliant blue



**Fig. 1. Wings, scale lattice, scatterograms and reflectance spectra of four representative *Morpho* species.** (A,E,I) *M. marcus*; (B,F,J) *M. achilles*; (C,G,K) *M. zephyritis*; (D,H,L) *M. aega*. (A–D) Photographs of the left dorsal wings. (E–H) Dark-field microphotographs of small areas of the wings. Arrowheads indicate cover scales; asterisks indicate ground scales. Scale bar: (E–H)  $200\ \mu\text{m}$ . In E and F, a few cover scales are lacking, so that the ground scales are exposed. (I–L) Scatterograms of an intact wing piece. (M) Wing reflectance spectra of the four species measured with an integrating sphere.



wings, which vary in hue and saturation (Fig. 1A–D). With bright-field epi-illumination microscopy, the dorsal wing scales display an intense blue reflection (Fig. S1). However, in Fig. 1E–H we show dark-field micrographs because more details of the scale organization are then revealed.

With dark-field illumination, the wing scales of *M. marcus* are multi-colored (Fig. 1E). Removing a few scales from a small wing area showed that the lattice of cover scales completely overlaps the lattice of ground scales. Whereas the cover scales are large, colorful and wrinkled (Fig. 1E, arrowhead), the ground scales are brown, small and more or less flat (Fig. 1E, asterisks). *Morpho achilles* has a similar scale organization, with very large cover scales completely overlapping the smaller ground scales; the color of both scale types is blue–greenish (Fig. 1F). In *M. zephyritis*, the scale arrangement is rather different. Here, the cover scales are slender, and their overlap with the strongly blue-reflecting ground scales is minor (Fig. 1G). The ground scales hence play a prominent role in the blue coloration of the wing. The case of *M. aega* is even more extreme, as only ground scales seem to be present; yet, minute cover scales can be occasionally discerned (Fig. 1H, arrowheads).

To better understand the contribution of the wing scales to the butterflies' visual display, we performed imaging scatterometry. We therefore took small wing pieces and mounted them, glued to a micropipette, in our scatterometer, and illuminated them with a narrow aperture light beam from a direction approximately normal to the plane of the wing piece. Interestingly, in the cases of *M. marcus* and *M. achilles*, the scatterograms showed two distinct, line-shaped patterns (Fig. 1I,J), but with wing pieces of *M. zephyritis* and *M. aega*, a single, very intense linear pattern was obtained (Fig. 1K,L). We explain below how we interpret the different spatial characteristics.

The micrographs (Fig. 1E–H) as well as the scatterograms (Fig. 1I–L) feature different colors. To investigate that in more detail, we measured the wing reflectance spectra of the four studied species with an integrating sphere (Fig. 1M). *Morpho achilles* wings have a smooth, blue-peaking reflectance spectrum, resembling that of a thin film (see e.g. Giraldo and Stavenga, 2016). The wings of *M. zephyritis* and *M. aega* have virtually identical reflectance spectra, with minor oscillations in the violet wavelength range. The reflectance profile of *M. marcus* slightly deviates, as it is shifted towards shorter wavelengths.

In order to unravel the coloration mechanisms and the microscopic structures responsible for the observed optical effects, we isolated single scales by gently detaching them from the wing and gluing them to a glass micropipette. Below we describe the scale properties of the selected species.

### Group 1: the coloration of *M. marcus*

The large cover scales of *M. marcus* are brilliant blue. Applying bright-field microscopy reveals that both the abwing and adwing sides have extremely wrinkled surfaces (Fig. 2A,B). The strongly undulated surface evidently caused the multiple colors of the scales when observed with dark-field microscopy (Fig. 1E). Compared with the cover scales, the ground scales of *M. marcus* are much smaller and rather flat (Fig. 2C,D). Their abwing side shows a non-uniform blue color (Fig. 2C), but the adwing side is brownish (Fig. 2D), which suggests the presence of melanin in the lower lamina of the scale.

We subsequently studied the scales' spatial reflection properties with the scatterometer. Approximately normal illumination of a small area of the cover scales with a narrow aperture beam evoked in both the abwing and adwing sides a blue, line-shaped scatterogram (Fig. 2E,F). In the case of the ground scales, their abwing side also

produced a line-shaped scatterogram (Fig. 2G), but the adwing side generated a more spatially restricted reflection pattern (Fig. 2H). The duller color of the latter scatterogram resembled the brownish color of the ground scale's adwing side (Fig. 2D).

For better insight into the observed spatial reflection patterns, we performed scanning electron microscopy (SEM) of both cover (Fig. 2I,J) and ground scales (Fig. 2L,M) of *M. marcus*. The upper lamina of the cover scales appeared to consist of rather thick and very closely packed ridges, with spacing of  $\sim 0.6 \mu\text{m}$ , connected by a thin membrane (Fig. 2I,J). As is seen from an oblique cut (Fig. 2J), the cover scales' lower lamina is locally rather flat, but as shown by the light micrographs, the total scale surface has severe undulations. The ground scales have ridges similar to those of the cover scales, but the space between the ridges and lower lamina is filled with irregularly organized membranes (Fig. 2L,M).

The anatomy readily explains the obtained scatterograms. The line-shaped far-field reflection pattern of the cover scale (Fig. 2E,F) may be understood from the distinct wrinkles in the scale surface that run parallel to the scale's longitudinal axis (Fig. 2A). However, we have to recall that a parallel array of longitudinal ridges generally acts as a diffraction grating, which creates a line-shaped scatterogram (see e.g. Stavenga et al., 2009; Giraldo and Stavenga, 2016). This holds for the abwing sides of both cover and ground scales (Fig. 2E, G,I,L). The scatterogram of a flat lower lamina is approximately dot shaped (Stavenga et al., 2009; Giraldo and Stavenga, 2016), but slight deviations of flatness causes widening of the scatterogram, as is seen in the adwing scatterogram of the ground scale (Fig. 2H).

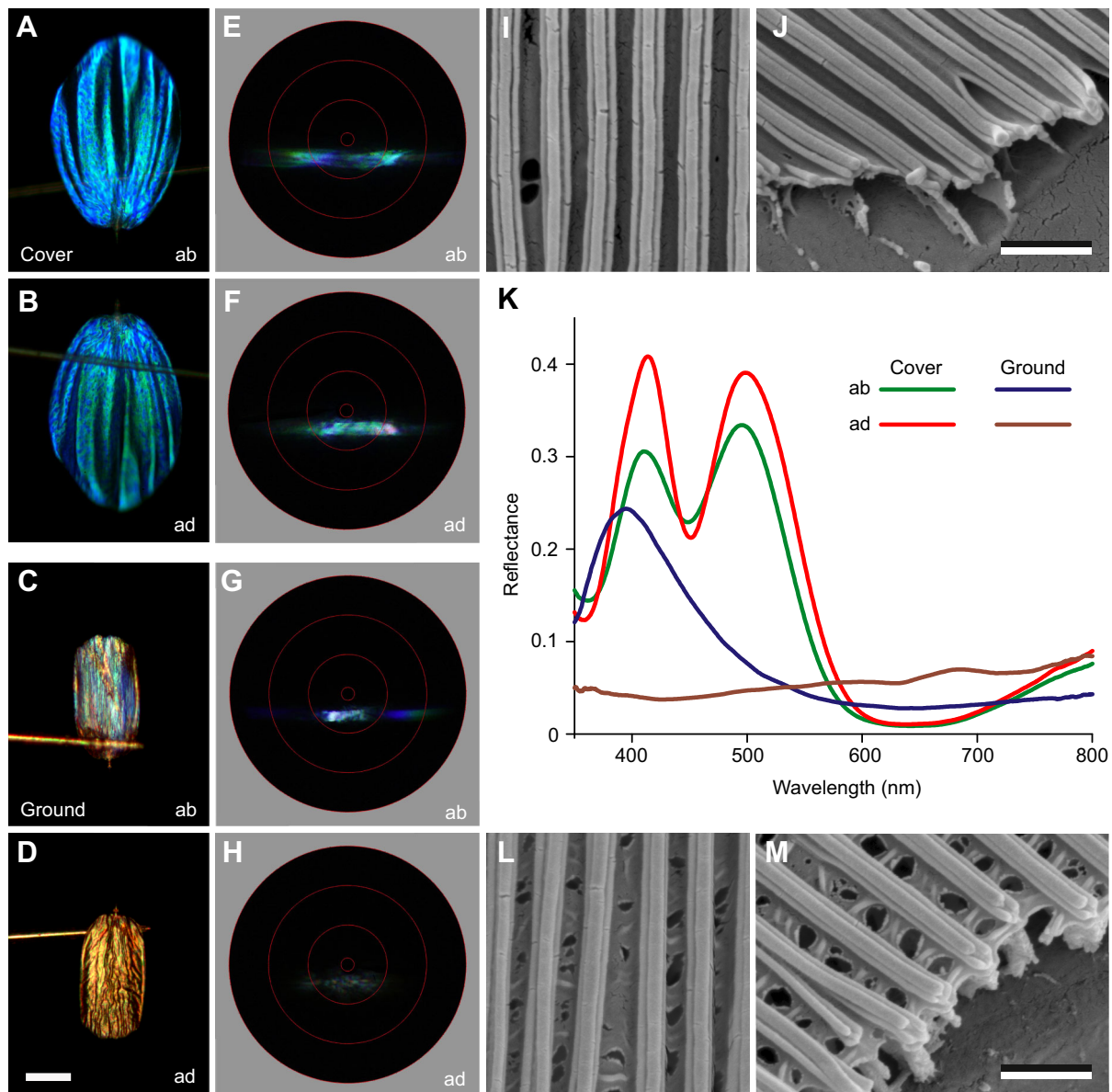
We also measured reflectance spectra with an MSP. The spectra of the abwing and adwing sides of the cover scale have two peaks, at  $\sim 420$  and  $\sim 500$  nm (Fig. 2K), which must result from light interference in the assembly of scale structures, i.e. the folded ridges, the membrane in between the ridges, and the thin film lower lamina. Very different reflectance spectra were obtained from the abwing and adwing sides of the ground scale. The abwing side yielded a simple blue–violet peaking curve (Fig. 2K), apparently owing to the scale's regularly structured upper side (Fig. 2L,M). The adwing reflectance was low and almost wavelength-independent, which suggests the presence of concentrated, broad-band-absorbing melanin pigment in the lower lamina (Fig. 2K). The ground scale's upper lamina is clearly unpigmented (see also Vukusic et al., 1999).

### Group 2: the coloration of *M. achilles*

The two sides of a cover scale of *M. achilles* observed with epi-illumination microscopy show similar blue–greenish colors; the abwing side is slightly dull, while the adwing side is brighter (Fig. 3A, B). The ground scales have two very different faces, similar as in the ground scales of *M. marcus*; the abwing side is bright blue–greenish (Fig. 3C), but the adwing side is blue–brown (Fig. 3D).

The scatterograms obtained from both sides of the cover and ground scales were intriguingly different (Fig. 3E–H). Notably, the scatterogram of the abwing side of the cover scales consisted of two lines, one resolving into dots (Fig. 3E). The scatterogram of the adwing side showed only a clear, bright spot (Fig. 3F).

To interpret the scatterograms we performed SEM. The cover scales had the usual array of ridges, but they were widely spaced and very sparsely connected by cross-ribs, thus leaving very open windows (Fig. 3I). The upper lamina rests on the lower lamina, with very thin and short trabeculae. The lower lamina, well visible through the windows, appeared to be extremely flat, suggesting that it will act as an almost ideal thin film. Indeed, the reflectance spectrum measured from the adwing side (Fig. 3K) closely approximates that of a chitinous thin film with a thickness of

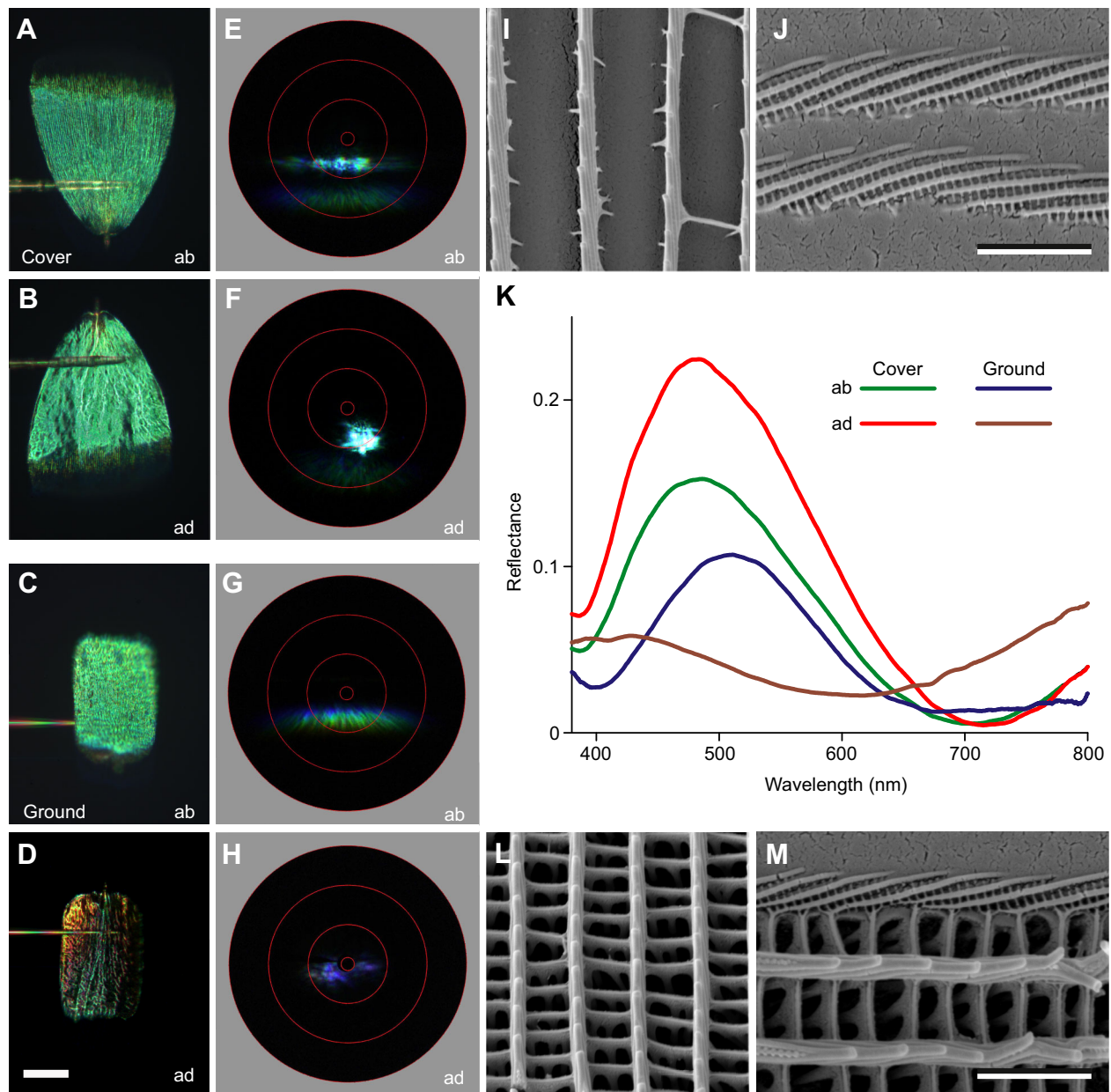


**Fig. 2. Optics of *M. marcus* scales.** (A–D) Abwing (ab, upper side) and adwing (ad, lower side) views of a cover scale (A,B) and a ground scale (C,D). (E–H) Scattergrams of the abwing and adwing sides of the cover (E,F) and ground scale (G,H). (I,J) Scanning electron micrographs of a cover scale with a perpendicular view in an undamaged area (I) and an oblique view of a sectioned area (J), showing its closely packed ridges and a rather flat lower lamina. (K) Reflectance spectra of the abwing and adwing sides of both cover and ground scales. (L,M) Scanning electron micrographs of a ground scale showing an intricate structured lumen between the ridge layer and the lower lamina. Scale bars: (A–D) 50  $\mu$ m; (I,J,L,M) 1  $\mu$ m.

240 nm, as holds for many other nymphalid butterflies (Stavenga, 2014; Stavenga et al., 2014; see also fig. 2 of Giraldo and Stavenga, 2016). Virtually the same reflectance spectrum was measured from the abwing (upper) side. The latter's slightly lower magnitude is understandable because of the scattering by the ridges. In fact, the array of sparsely spaced ridges together with the thin film lower lamina will act as a reflection grating. The ridge distance of 1.5  $\mu$ m predicts diffraction orders with angular distance 15 deg for 500 nm wavelength light, in full agreement with the separation of the spots in the dotted line of the abwing scattergram (Fig. 3E). The scattergram also showed a somewhat diffuse line, displaced by  $\sim$ 30 deg from the dotted line. This angle corresponds to approximately twice the angle of the ridge lamellae with respect to the lower lamina (Fig. 3J). The three to four overlapping lamellae create a very slender multilayer, which causes a diffuse line in the

scattergram. The narrow ridge multilayer reflection has a similar, but much weaker color as that of the thin film lower lamina (Fig. 3E), and thus the latter will dominate the reflectance spectrum (Fig. 3K).

The ground scale scattergrams can be also well understood from the anatomy and spectral measurements (Fig. 3G,H,K–M). The ridges are spaced more closely than in the cover scales, but a dense array of cross-ribs notably connects the ridges, which again consist of stacked lamellae (Fig. 3L,M). The abwing scattergram is a diffuse line, caused by the ridge multilayer, which reflects mainly in the blue–green wavelength range (Fig. 3K). The adwing scattergram shows a spatially restricted bluish pattern, caused by a very slightly wrinkled lower lamina, acting as a thin film with a thickness of  $\sim$ 200 nm. The ground scale evidently contains ample melanin pigment, as indicated by both the adwing photograph and the reflectance spectrum (Fig. 3D,K).



**Fig. 3. Optics of *M. achilles* scales.** (A–D) Abwing and adwing views of a cover scale (A,B) and a ground scale (C,D). (E–H) Scattergrams of the abwing and adwing sides of the cover (E,F) and ground scale (G,H). (I,J) Scanning electron micrographs of cover scales (I, top view; J, side view). (K) Reflectance spectra of the abwing and adwing sides of both cover and ground scales. (L,M) Scanning electron micrographs of ground scales (L, top view; M, side view of a scale with a bent-over ridge on top). Scale bars: (A–D) 50 μm; (I,J,L,M) 2 μm.

### Group 3: the coloration of *M. zephyritis*

The optics of *M. zephyritis* scales differs substantially from that of the previous case. The cover scales have a much more slender shape than the ground scales (Fig. 4A–D), as was already seen in Fig. 1G. Whereas the abwing sides of both cover and ground scales are bright blue, the adwing sides are brownish (Fig. 4A–D), in all cases causing line-shaped scattergrams (Fig. 4E–H).

SEM yielded an almost identical anatomy of the cover and ground scales. They have rather closely spaced, tall ridges, made up of large stacks of lamellae (Fig. 4I,J,L,M), explaining the linear abwing scattergrams (Fig. 4E,G). The large number of overlapping lamellae, ~10 in both the cover and ground scale ridges, creates tall multilayers. The reflectance spectra, which are virtually identical, are blue-peaking and have much narrower bandwidths

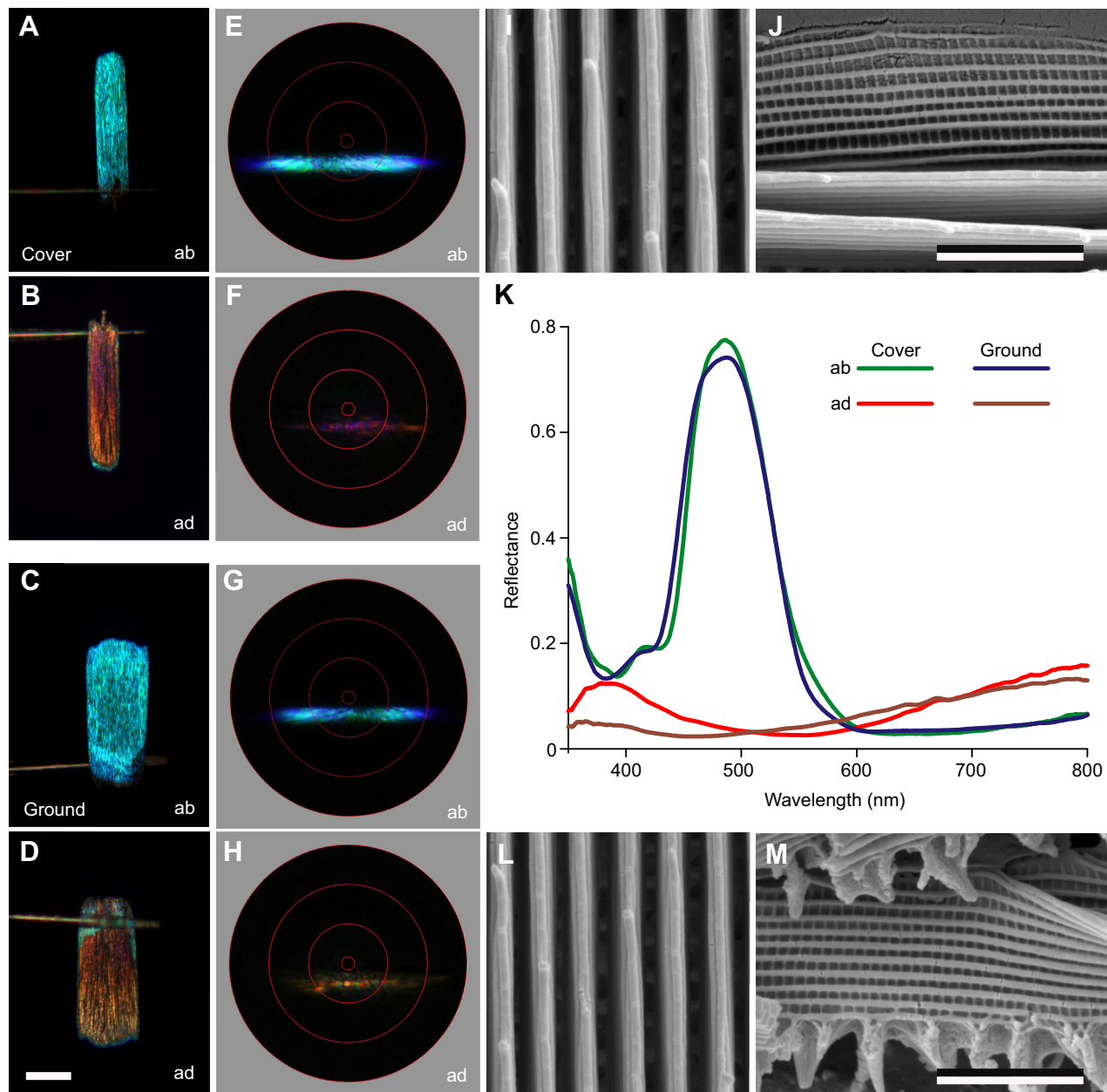
than the reflectance spectra of *M. achilles* (Fig. 4K). This is intimately connected with the difference in stacked lamellae.

The adwing scattergrams of the cover and ground scales are line-shaped because of the non-flat, crinkled lower lamina (Fig. 4B,D,F,H). Their brownish color demonstrates the presence of a substantial amount of melanin, but the reflectance spectra also indicate thin film lower laminae (Fig. 4K).

### Group 4: the coloration of *M. aega*

The intense blue wings of *M. aega* are covered by virtually only ground scales (Fig. 1H), which have a bright blue abwing side and a brown adwing side (Fig. 5A,B,F), similar to the ground scales of *M. zephyritis* (Fig. 4C,D). The scattergram of the abwing side of a ground scale (Fig. 5C) shows a bright blue line, and the pigmented





**Fig. 4. Optics of *M. zephyritis* scales.** (A–D) Abwing and adwing views of a cover scale (A,B) and a ground scale (C,D). (E–H) Scatterograms of the abwing and adwing sides of the cover (E,F) and ground scale (G,H). (I,J) Scanning electron micrographs of cover scales [I, top view; J, side view of a scale with one ridge ‘bent over’ (top)]. (K) Reflectance spectra of the abwing and adwing sides of both cover and ground scales. (L,M) Scanning electron micrographs of ground scales [L, top view; M, side view of a scale with (again) one ridge bent over (as in J)]. Scale bars: (A–D) 50  $\mu$ m; (I,J,L,M) 2  $\mu$ m.

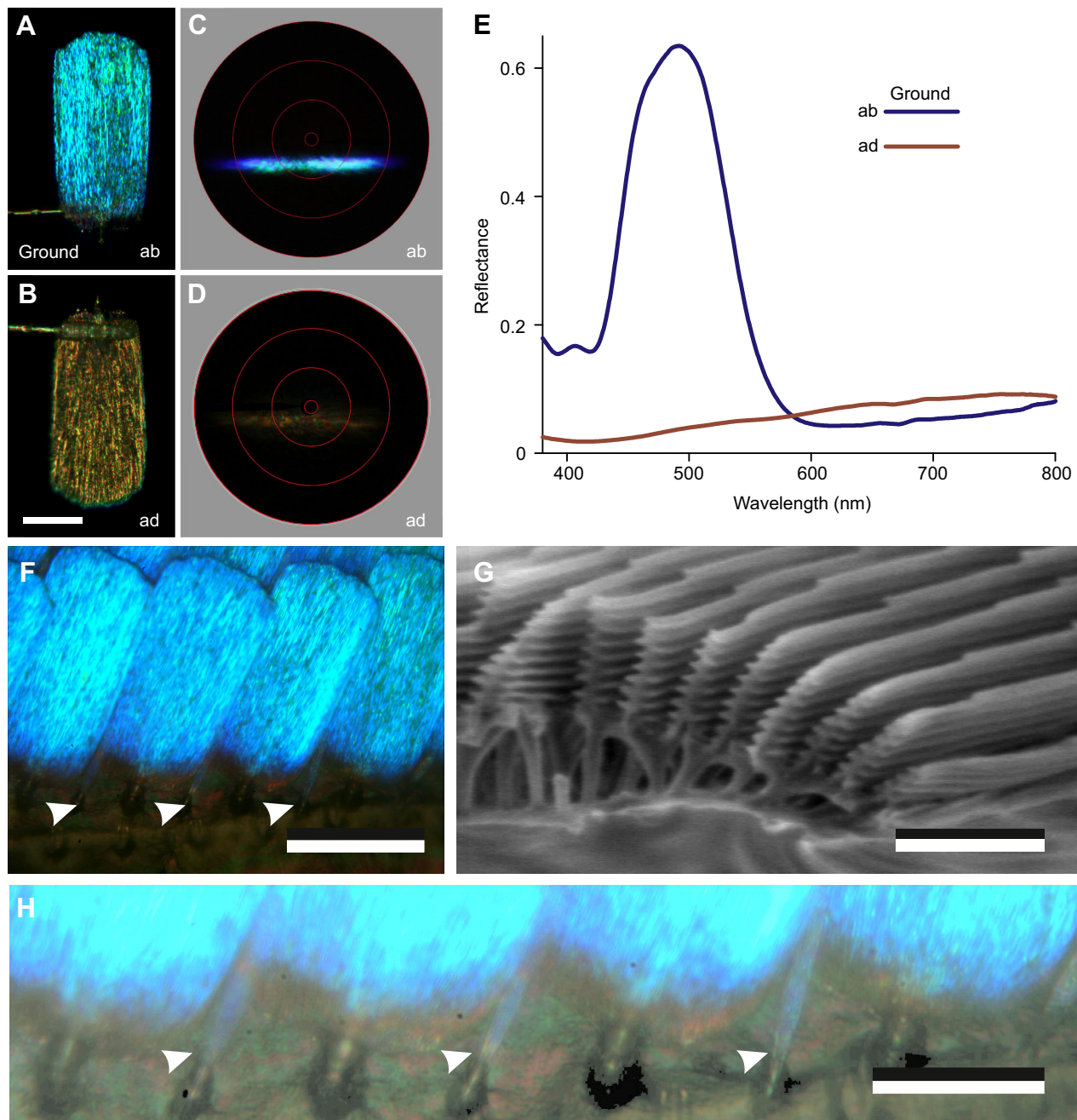
lower lamina generates a faint brown line (Fig. 5D). The abwing’s bright blue reflection is caused by the large stack of lamellae (10 layers) of the upper lamina’s ridges (Fig. 5G). The abwing reflectance spectrum has a clear peak in the blue wavelength range (Fig. 5E), very similar to the abwing spectra of the cover and ground scales of *M. zephyritis* (Fig. 4K). The faint brown adwing scatterogram corresponds to the low reflectance, which increases gradually with increasing wavelength (Fig. 5E), demonstrating a substantial amount of melanin.

Close inspection of an area where some scales are removed reveals a perfectly regular array of cover scales (Fig. 5F,H). These scales are extremely short and slender, however, and therefore they are mainly or even completely covered by the much larger ground

scales. We note here that in those cases where only one scale type seems to be present, there is always a (sometimes indeed very small) minority of tiny scales in addition to much larger scales. We interpret these tiny scales to be cover scales because they are organized in a row close to and right behind the row of the larger scales. This characteristic layout of two adjacent rows is generally observed in Lepidoptera (front row for ground, rear row for cover); the fact that the larger scales are pigmented reinforces this interpretation.

#### Comparison of the scale properties of several *Morpho* species

We investigated in total 16 *Morpho* species along the lines presented above. Fig. 6A gives an overview of the scale patterning in the



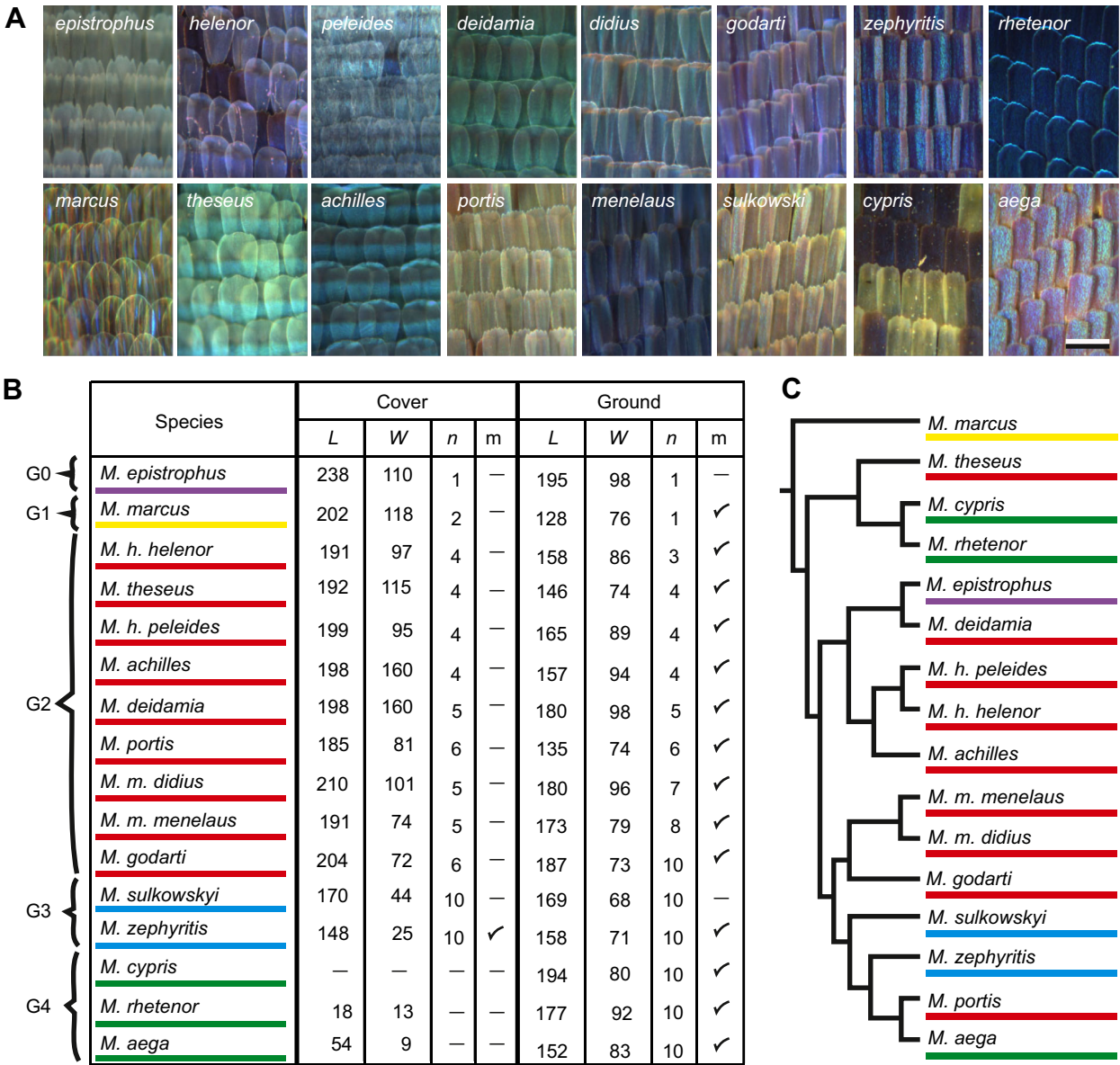
**Fig. 5. Optics of *M. aega* scales.** (A,B) Abwing and adwing views of a ground scale. (C,D) Corresponding scatterograms. (E) Reflectance spectra of the abwing and adwing sides. (F) Epi-illumination light microscopic photograph of ground scales with exposed roots, which normally are overlapped by other ground scales, here lacking; arrowheads point to the tiny cover scales. (G) Scanning electron micrograph of a sectioned scale, showing the multilayered ridges. (H) Magnified area of the scale root region of F, with arrowheads pointing to the cover scales. Scale bars: (A,B) 50  $\mu$ m; (F) 2  $\mu$ m; (G) 100  $\mu$ m; (H) 50  $\mu$ m.

investigated species as seen with dark-field, epi-illumination microscopy (corresponding bright-field micrographs are shown in Fig. S1). Furthermore, for each species we present for both the cover and ground scales the length and width, the number of the ridge lamellae and the presence (or absence) of melanin. We arranged the species according to ascending number of lamellae of the ridges of the ground scales; the number of ridge lamellae of the cover scales increased in virtually the same order (Fig. 6B). Similar values were reported by Berthier et al. (2006).

We distinguished five groups according to the relative size of the cover and ground scales and their degree of overlap (Fig. 6B, G0–G4). In the single species in G1 (yellow underline in Fig. 6B,

*M. marcus*), the cover scales are large and completely overlap the ground scales. It is singled out because its scale structure deviates from that of the other *Morpho* species. In the species in G2 (red underline), the cover scales are larger than the ground scales and the overlap is considerable. The species in G3 (blue underline) have slender cover scales, which only slightly overlap the ground scales, and in the species in G4 (green underline), the cover scales are minute or even absent. Fig. 6C shows the (condensed) phylogenetic tree conceived by Blandin and Purser (2013) restricted to the species that we studied. Comparison of our groups with the phylogenetic tree shows a general agreement, but also some clear discrepancies.





DISCUSSION

We arranged the 16 investigated *Morpho* species according to the number of lamellae of the wing scale ridges, and distinguished five groups. We placed *M. epistrophus* in the basic group G0 because its scale organization is very similar to that encountered generally in nymphalids. We divided the other species into four additional groups using the degree of overlap of cover and ground scales as a distinctive criterion: from a complete overlap in G1 to a full dominance of the ground scales in the species of G4. In the latter group, the cover scales are either absent or strongly atrophied. G2 and G3 are intermediate states in a progressive reduction of the cover scales. The ordering of the *Morpho* species on the basis of scale properties largely corresponds to that of the phylogeny derived on the basis of biographical considerations (Blandin and Purser, 2013), although some distinct discrepancies cannot be neglected

(Fig. 6C). Presumably, local conditions resulting in evolutionary pressure to enhance display will favor scales with tall stacks of lamellae. As demonstrated by the scattergrams, the superposition of cover and ground scales at the intact wings (Figs 1I–L and 6A) has important consequences for the spatial reflection patterns. Usually, the optics of *Morpho* butterflies is treated as if the bright blue, iridescent coloration is solely determined by the stacked ridge lamellae, together acting as a multilayer reflector. However, our comparative study revealed that the optics is often much more complex. The number of ground scale lamellae increases when scale overlap decreases (Fig. 6B), thus yielding an increasingly dominant role of the ground scales. In the basic case of *M. epistrophus*, the blue wing color is caused by light reflected by the lower laminae of both cover and ground scales, which is scattered by the upper

laminae, thus resulting in rather diffuse reflections (Giraldo and Stavenga, 2016).

Actually, *M. marcus* may be even more basic than *M. epistrophus*. Whereas in *M. marcus* the cover scales fully overlap the ground scales, in *M. epistrophus* the cover scales only partly overlap the ground scales (Giraldo and Stavenga, 2016). In *M. marcus*, the scale ridges are poorly developed and consist of only one lamellar layer. The blue wing color is here produced by both the upper and lower laminae, resulting in a unique reflectance spectrum with two peaks. Currently accepted phylogeny indeed locates *M. marcus* at the top of the *Morpho* clade, and thus it is considered to be most ancestral (Blandin and Purser, 2013).

On the other end of the chain is *M. aega* (G4), which belongs to the most derived group of the genus. In this species, the cover scales are severely reduced. The tall stacks of lamellar ridges on the ground scales cause a high and spectrally narrow-band scale reflectance, and incident light is reflected quite directionally. Possibly the loss of cover scales and the increased number of ridge lamellae in the more advanced species favor a selection of strong spatial signaling of the males to the females.

Yet, for *M. rhetenor* and *M. cypris*, the other two species that we classified as belonging to G4, other taxonomic traits do not locate them as derived as *M. aega*. In the phylogeny tree of Blandin and Purser (2013), they occupy a rather intermediate position, even before *M. deidamia*, *M. helenor* and *M. sulkowski*, members of G2 and G3. For the members of the intermediate groups, our division (Fig. 6B) and the phylogeny tree (Fig. 6C) agree reasonably well.

Classifications of *Morpho* butterflies on the basis of wing shape (important for flying behavior) put forward by DeVries et al. (2010) and Chazot et al. (2016) also somewhat deviate from the evolutionary trees based on molecular biology and biogeography. The present comparative study of several *Morpho* species extends previous findings, emphasizing that *Morpho* is a very diverse genus, with wing scales varying from the basic butterfly wing scale bauplan to the extremely specialized ‘Christmas tree’ scales. Further evo-devo studies will be necessary to solve the intriguing question of how the various structures have come into existence.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

All authors participated in the experiments. M.A.G. and D.G.S. wrote the manuscript, which was approved by all authors.

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#### Supplementary information

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